COMMENTS ON "ONE GENERATION" EXTENSION STUDYCONTRACT NUMBER: 68-W-01-023WORK ASSIGNMENT: 1-10, TASK 2BYDR. PAUL M.D. FOSTER

GENERAL COMMENTS

The proposed assessment of the value of adding extra animals to improve the ability to detect potential responses affecting the male reproductive development is an excellent initiative and Agency should be applauded for these efforts. I would counsel that this early initiative to study this important issue should be viewed a "proof of principle" endeavor. That is, it will provide fundamental information on the best use of F_1 animals (a precious resource and only obtained from the multigeneration assay in our standard repertoire of tests) with regard to the ability to detect hormonally mediated changes, particularly in reproductive development where certain questions have been raised with regard to the sensitivity of the current experimental design. This proposal should also provide valuable data on the utility of the standard necropsy of animals at pnd 21 and the information that may be derived from these activities. In this instance it will be important to ascertain the relative value of the study of weanling animals and could potentially direct efforts toward an extended evaluation of mature animals, where their reproductive tracts are more mature, as a substitution and thus be able to provide more definitive information for use in the risk assessment of endocrine active chemicals. The selection of compounds is very appropriate for this "proof of principle" experimentation as is the dose route selected. For other unknowns, exposure during lactation will become an important consideration and may require direct dosing of pups during this critical developmental window.

The proposed study design is fit for the purpose envisaged. The proposed experimentation should yield highly informative data on which to make decisions on the best use of animals to gain the maximum information. Again, I applaud the Agency in its attempts to bring the best science to bear in modifying and improving our testing paradigms.

Wherever possible I believe the developed protocol should embody those measurements, methods and times routinely undertaken by the contracting laboratory in their standard multigeneration reproduction studies. This will have at least two advantages: 1) the lab will be familiar with scheduling and conducting such events and 2) it will enable a direct comparison with historical control data for any particular end point within the laboratory's experience. For example, if anogenital distance is normally measured on pnd 0 in the lab (as it is in my own), it does not make sense to take this measurement on pnd2. The data obtained will be equivalent and some leeway should be provided to the investigator

I am concerned that the day of terminal necropsy of the animals is too soon (pnd 60), again this is not the time when these animals would normally be killed in a multigeneration study and also is on the cusp of sexual maturity for the SD rat. I would suggest that this time be pushed back at least 10 days and preferably to pnd 90. This will then allow greater flexibility in the timing of the necropsies. Organ weights, etc. will be little different between say a 90 and 95-day animal, whereas a 2-3 day difference at pnd 60 could be crucial. One should bear in mind that the proposal would indicate that there may be some 350+ animals to necropsy at this time

and we would not want to make this activity unduly cumbersome. Similarly, the request for necropsy of these 350+ animals within a 2-hour period, over 2 days is likely to prove impossible even for the best laboratories. Clearly the circadian rhythms of hormone secretion are an important contributor to potential variability in these measurements. On the other hand, changes in hormone measurements, in the absence of structural and functional changes are frequently not sensitive when only taken at one time per animal. I would suggest using a cohort of the animals (1 to 2 per litter) to be necropsied within the specified time range on the designated necropsy days. However, I would also advocate taking terminal blood samples on all animals for hormone analysis. If major changes are present they should still be evident in these samples. Subtle changes would probably be restricted to the timed cohort animals.

I am greatly enthused by the potential data that could arise from this project and would be extremely pleased to be able to contribute and provide input into the final protocol study design and evaluation of the data on completion of the study.

SPECIFIC COMMENTS

1. Animals. This strain is appropriate for these experiments. The objective should be to obtain 20 litters for examination. Given the good pregnancy record of the SD rat, it may still be prudent to add a few extra pregnant dams to ensure 20 litters.
2. Litter size. I am not an advocate of culling litters and this has been a contentious issue in reproductive toxicology for many years. I would suggest that we should use the laboratory's normal practice for doing this randomly with the exception that the number of males should be maximized.
3. Chemicals and dose levels (see later)
4. Dosing route. Appropriate for "proof of principle" and with the background information that is available for the 2 test chemicals using this route of exposure. At some later time a feed and/or drinking water study would also be appropriate. However with this relatively short duration of exposure and the knowledge of the changes in food and water consumption that occur during pregnancy and lactation the gavage route does afford better ability to control delivered dose.
5. Dosing duration. Appropriate
6. Sacrifice. I would prefer pnd 90 rather than pnd 60 – see above.
7. End points
   a) Maternal. Use normal days for multigen study employed by lab for bodyweight and food consumption.
   b) Neonates. AGD day should be as normal for lab. I would suggest adding survival indices for pups (e.g. pnd 4, 7, 14 and 21) and not just litter size at birth. We would thus have data on litter size and sex ratio at times other than the day of birth. Keep standard multi gen days for body weight and food consumption.
   c) Female cohort –OK
   d) Male cohort – OK but with change of necropsy date. It is not clear what will be measured at the pnd 21 sacrifice versus the F_1 adults. The different lobes of the prostate will be a significant challenge at pnd 21. Likewise, the seminal vesicles will have no fluid in weanlings. Hypospadias can be detected in weanlings, but it is significantly more difficult than as an adult.

e) Specific instructions. I have already made comment on the time allotted for necropsy as being too short and the potential use of a cohort for hormone analysis. Testes and epididymides should be weighed separately (unilateral effects are observed on a regular if infrequent basis) Allow the use of the lab standard methods normally employed for sperm and spermatid counts, providing it produces equivalent data

COMPLETENESS OF LIST OF END POINTS

The list is very comprehensive. I would add the *levator ani bulbocavernosus* muscle weight at necropsy of the F_1 adult males. It is androgen dependent and provides some cross correlation with some tier 1 measurements.

DOSE LEVEL SELECTION OF TEST COMPOUNDS

Good choice of agents and dose levels. We should produce clear-cut effects at the highest dose level and more marginal changes at the lowest. A good test for sensitivity.

OTHER POTENTIAL AGENTS

In general "blockbuster" chemicals are not going to present a problem for an enhanced multigeneration reproduction study. Subtle effects and good dose response data for risk assessment are the area where increased ability to detect change will come to the fore. I would suggest selecting agents based on mode of action and potency.

ANDROGEN RECEPTOR ANTAGONISTS

Procymidone and a low dose of flutamide will cover potent antagonists. Linuron represents the weaker agonist activities as does p.p'-DDE.

STEROID BIOSYNTHESIS INHIBITORS

Di-iso-nonyl phthalate (DINP) is one of the weaker acting esters and could be a good test agent (particularly as the published multigeneration study (Waterman, S. J., L. H. Keller, G. W. Trimmer, J. J. Freeman, A. I. Nikiforov, S. B. Harris, M. J. Nicolich & R. H. McKee: Two-generation reproduction study in rats given di-isononyl phthalate in the diet. *Reprod Toxicol* 2000, 14:21-36.) is negative) to compare to DBP.

OTHER AGENTS

I think the use of an enzyme inducer would provide some useful information. Phenobarbital has been reported to affect reproductive development by increasing the clearance of sex steroids (this may require an exposure period before breeding to achieve induction during the critical windows of reproductive development.). A 5α reductase inhibitor (such as finasteride) could provide other data for a mode of action known to affect male reproductive development.